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GASEOUS EXCHANGE IN A CLOSED ECOLOGICAL SYSTEM

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FOREWORD

This work was accomplished in support of Project No. 7164, "Space Biology Research," Task No. 716403, "Environmental Biology," by the Biospecialties Section, Physiology Branch, Biomedical Laboratory, 6570th Aerospace Medical Research Laboratories. The research was accomplished between April 1961 and June 1962.

The experiments reported herein were conducted according to the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

ABSTRACT

A series of experiments was performed to determine the feasibility of using several types of organisms to maintain a gaseous balance in a closed system. The system consisted of: (a) a photosynthetic unit comprised of cells of Chlorella pyrenoidosa strain TX 71105, (b) a biological aerobic sewage-digesting unit, (c) a unit containing an ammonia-utilizing fungus, Linderina pennispora, and (d) an animal chamber containing a white adult male rat. Although many difficulties were encountered, this bioregenerating unit could be operated under closed conditions for 390 hours. Gas analyses indicated CO_2 and O_2 levels can be maintained at desirable levels. Material balance within the system was not attempted. However, nitrogen analysis of the resultant fungal mass showed that data on the basic physiology of the organism is required before such a balance can seriously be attempted. The relationship between mission requirements and the level of biological complexity of a closed ecological system was discussed.

PUBLICATION REVIEW

This technical documentary report has been reviewed and is approved.

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INTRODUCTION

The extension of man's environment to include outer space requires that his biologistic support be increased both qualitatively and quantitatively. Although the basic requirements—food, water, sanitation, breathable atmosphere, etc.—have always been a problem in both old and new environments, the introduction of man into space for extended time periods necessitates the consideration of problems much more complex. This complexity results from the severe restrictions of weight and space which any space vehicle or station will encounter. The biologistic problem may then be simply stated: "to provide man with reliable means of satisfying his physiological requirements within the confines of a limited ecosphere."

The support of biological entities is an endeavor to, thermodynamically speaking, maintain the status quo of the entropy of each of the subsystems comprising the ecosphere, or at least to control its increase at acceptable rates. Energy will be put into the system via oxidizable nutrients and other physical means such as solar, nuclear, etc. The space ecologist must then manipulate this energy in such a way as to make it available to the organisms within the closed system. What this really means is, assuming energy will not be the limiting factor, that the conservation of the elements—carbon, oxygen, nitrogen, hydrogen, etc.—which are in reality media by which energy is made available, is the critical limiting factor. Since a continual resupply of these elements in the form of food, water, etc., cannot be accomplished, these molecules must be re-used at a high rate of efficiency, that is to say, a high rate of recovery.

The biologistic problem which has probably received the greatest attention is that concerning the establishment of a habitable gaseous environment. Primarily involved are CO_2, O_2, and the variety of noxious gases arising as metabolic waste products. The arguments concerning various chemical and physical processes need not be reiterated. On theoretical grounds, bioregenerative, i.e., photosynthetic, techniques are at the very least competitive. In addition, material is synthesized, viz. protoplasm, which can be further utilized to facilitate the flow of energy and elemental substances. Evidence (ref. 18) also indicates contaminants other than CO_2 may be removed from the atmosphere by photosynthesizing algae. We shall, therefore, consider the CO_2-O_2 problem to be resolvable via the photosynthetic unialgal system.

A second factor requiring bioengineering attention is the removal of fecal, urinary, and other wastes. Besides a great portion of water, these substances are comprised of a mixture of biochemical entities which might be used as energy sources and essential nutrients for particular bioforms within the system. The removal of sewage from the ecosphere is then just half of this problem. The other half concerns the processes and techniques by which this material must be treated to permit continuation of the closed ecological system. The biological approach to sewage digestion results in the formation of sewage effluent with a low Biochemical Oxygen Demand (B.O.D.)—i.e., an oxidatively stable milieu—and the reproduction of those microbiological entities responsible for this transformation. In addition, certain gaseous products are formed to include ammonia and carbon dioxide.

A considerable amount of effort has been devoted to the study of using "sewage nutrients" for algal growth (refs. 1, 4, 7, 10, 13, 15, 31, 32, 34, 35, 37). These studies have indicated that algae can utilize certain substances present in sewage via heterotrophic pathways. Conversely, so-called sewage ponds, which consist of an intimate mixture of algae and sewage organisms, provide a rather simple means of sewage stabilization. Apparently, the photosynthetic organisms are effective by providing a highly aerobic environment for the activities of the sewage-digesting organisms. Work in this and other laboratories has shown that heterotrophy or heterotrophy plus autotrophy may occur (so-called mixotrophy) and hence the algae may also contribute directly to the sewage degradation (refs. 2, 3, 5, 6, 9, 17, 21, 23, 24, 26, 27, 29, 30, 33, 34, 39-41).

As previously mentioned, ammonia is a product of aerobic sewage degradation arising from the proteolytic activities of the sewage organisms. This gas might be a good source of nitrogen for photosynthesizing organisms. Various workers (refs. 21, 30, 38, 39, 42) have studied the availability to algae of nitrogen as nitrate and as ammonia, the data being somewhat conflicting. In all of these studies the nitrogen was supplied as a salt. Attempts to employ gaseous ammonia as the nitrogen source have not appeared in the literature. However, Gray* has shown that the fungus, Linderina pennispora, can utilize gaseous ammonia. Undoubtedly it appears in the medium in the ionic form. Gray also has shown this organism to synthesize a large quantity of protein which appears to be a tolerated food supplement for rats. In addition, he has used lyophilized algal cells as a carbon source for the fungus and has obtained a conversion efficiency of 20 percent.

Considering the four groups of organisms discussed above—i.e., animals, algae, fungi, and "sewage organisms"—one can detect a metabolic thread which could tie these diverse forms together. If one examines the waste products and the nutritive requirements of these groups, individual substances—e.g., CO_2, O_2, NH_3, feces, etc.—can be found in both categories. In this investigation we studied the feasibility of establishing a symbiotic relationship among the above organisms as diagrammed in figure 1. Only the first phase, that of gaseous balance, is described in this report.

MATERIALS AND METHODS

A laboratory unit was constructed which consisted of: (a) an illuminated algal chamber, (b) a fungal chamber, (c) an aerobic sewage digestion chamber, (d) an animal chamber, and (e) accessory filters, condensors, pumps, and connectors. The unit is depicted in figures 2 and 3.

Bioregenerating Unit (BRU)

A 4-liter serum bottle, with outlet, measuring 30.5 cm in height and 16.5 cm in diameter, was used as the algal growth chamber. A total of 2.5 liters of medium was added to the bottle and a rubber stopper was used as the closure. Sufficient ports through the stopper were present to enable

* Gray, W. D, Personal Communication, Ohio State University, Columbus, Ohio, 1961.

continual passage of air and return of condensed water. The chamber was connected to glass wool filters, a water scrubber, and a condenser (as shown in figure 2), and sterilized as an intact unit. The chamber was illuminated by 3 General Electric Circline fluorescent lamps (FC12T10-CW), or equivalent, which surrounded the vessel at 3.5-inch intervals. When centrally located within the lamps, the outer wall of the chamber was approximately 1 inch from the inner face of the lamps. The lamps were supported by an aluminum shield 12 inches high, which served as both a light reflector and a heating jacket, the heat from the lamps maintaining the temperature between 37° and 39° C.

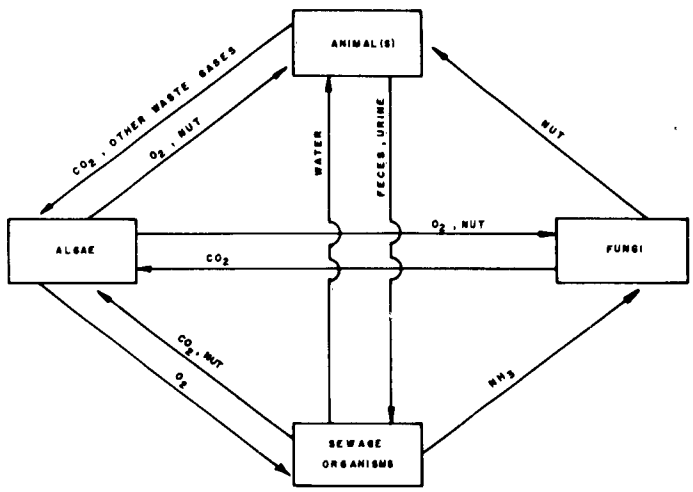

Figure 1. Diagram of a Closed Ecological System

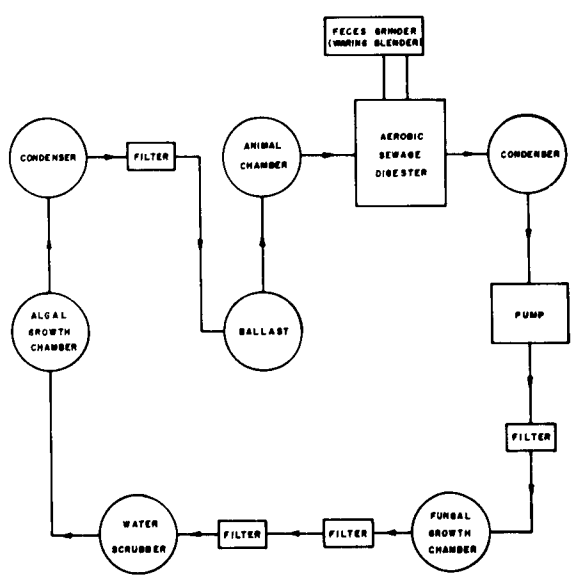

Figure 2. Diagram of the Bioregenerating Unit

Figure 3. The Bioregenerating Unit

A 4-liter serum bottle containing 2.5 liters of medium was also used as the fungal growth chamber. The vessel was closed with a rubber stopper fitted with gassing tubes and sparger, and glass wool filters were attached. The entire unit was then autoclaved at 15 pounds pressure for 15 minutes. The sterile algal and fungal chambers were then connected to the system as shown in figure 2.

The sewage chamber consisted of a Lucite container measuring approximately 30 by 30 by 30 cm. The lower half was given a V-configuration with an angle of about 100°. This enabled continual mixing of the sewage without using any scraping device. Air was bubbled through the sewage via a 0.63-cm-diameter perforated stainless steel tube which was placed at the bottom of the V-shaped trough. Provisions were made for the addition and withdrawal of fecal and sewage samples and the return of condensed water. Eight liters of distilled water was added which gave an air head in the chamber of about 13.5 liters. The air space in the total system was estimated to be 29 liters. This would give a composition of approximately 23.1 liters of N_2, 5.7 liters of O_2, and not more than 0.2 liter of CO_2 (as analyzed by gas chromatography). The animal chamber was a Lucite box measuring 15 by 17 by 20 cm. The animal in the chamber had continual access to food and water. Provisions were made for adequate air circulation and air sampling of the effluent air stream. Feces and urine accumulated during the experiment. Production of NH_3 under these conditions would serve as an additional nitrogen source for the fungi. The feces used in the sewage digestion chamber were obtained from other animals during the course of the experiment.

Feces were added to the sewage chamber by first drawing effluent from the chamber to a modified Waring blender cup using a separate vacuum system. Rat or rabbit feces were then placed in the cup and homogenized. The mixture returned to the sewage chamber by gravity flow, without permitting air to be introduced from the miniature comminutor. The amounts of feces added were varied during the experiment. In all cases we used high concentrations of solids, averaging about 10,000 ppm. Since a batch basis approach was involved, the chamber was operated under varying load conditions.

The atmosphere within the system was continually circulated through all units by a rubber diaphragm pump (Neptune Dyna-Pump). This pump worked quite well as grease seals were not involved. The pump operated under the system load quite adequately.

Changes in air volume due to repeated sample withdrawals (for gas analyses) were compensated for by a water ballast.

Media

A modified Knops medium (MS) of the following composition was used for the support of the algal cells: KNO_3, 1.21 grams; $MgSO_4 \cdot 7H_2O$, 2.46 grams; KH_2PO_4, 1.23 grams; $Na_3C_6H_5O_7 \cdot H_2O$, 0.195 gram; iron and ammonium citrate, 0.0853 gram; $Ca(NO_3)_2 \cdot 4H_2O$, 0.02 gram; EDTA, 4 mg; Arnon's A5 trace element solution, 1 ml; distilled water, 1 liter. Without an additional carbon source this medium will not support significant amounts of algal growth. Dark experiments have shown the citrate levels were not adequate to provide a heterotrophic source of carbon.

The fungal medium had the following composition: corn steep liquor, 2 ml; D-glucose, 10 grams; NH_4Cl, 1 gram; KH_2PO_4, 2.5 grams; 4.4 percent weight per volume of $ZnSO_4$ solution, 1 ml; distilled water, 1 liter. A few drops of corn oil were added as an antifoam agent.

Organisms

The alga used in this experiment was the high-temperature Sorokin strain of Chlorella pyrenoidosa, TX 71105. Stock cultures of the cells were maintained by thrice weekly transfers in modified Knops medium containing 0.5 percent glucose. The inoculum for the growth chamber consisted of 5 ml of a 48-hour culture. This yielded an initial cell concentration of approximately 1×10^6 cells per ml. Cell densities were determined by microscopic counts using a hemocytometer. Samples of the algal population were removed aseptically for cell density and sterility determinations.

Stock cultures of Linderina pennispora were maintained on agar slants of the medium previously described with substitution of 7 grams of yeast extract for the corn steep liquor. After streaking, the slants were incubated at room temperature until sporulation had occurred. The slants were then stored in the refrigerator until required. Spores from one slant were washed off with sterile water and used as the inoculum of the fungal growth chamber. L. pennispora exhibits a wide temperature tolerance and hence the fluctuations in room temperature were accepted as satisfactory for growth conditions. Fungal yields were determined terminally by weighing the filtered, washed, and lyophilized mycelial pad.

Gas Analyses

The gases of prime interest were O_2, N_2, CO_2, and NH_3. Due to the small amounts of available NH_3 in the closed atmosphere at any one time and the small volume of the sample (10 ml), an adequate method for NH_3 determination has not been found. The remaining gases were measured with the Perkin Elmer Vapor Fractometer Model 154. A parallel column adaptor was employed enabling the simultaneous use of both silica gel and molecular sieve columns (Perkin-Elmer columns I and J, respectively) with a single detector. A 0.5-meter silica gel and a 2.0-meter molecular sieve were used at 32° C. with the helium carrier pressure at 5 pounds (inlet) and a flow rate of 95 mm at the outlet.

The gas concentration was estimated quantitatively by determining the areas under the peaks by planimetry or by applying the formula:

$$a = hw_2$$

where a is the area, h the peak height, and w_2 the peak width at 1/2 h. The latter method was shown to be as reliable as the former and has the advantage of being much less time consuming. Only CO_2, N_2, and O_2 were detected and measured. Percent composition as presented, therefore, does not include other trace gases. Certain difficulties were experienced with the gas chromatograph due to improper packing of the columns. Subsequently, all gas analyses were then performed with the Haldane apparatus.

RESULTS

The variability in the experimental procedure for chromatographic gas analyses was determined by measuring the gas composition of room air several times on various days. Table I shows the observed variation. Results were calculated by planimetry. Examination of the standard deviations for these values shows good agreement of the results obtained from day to day analyses. Table II shows the gas composition of a trial run which included all the microbial entities but not the animal. Comparison of the standard deviations of these values with those of the data in table I indicates that variation from port to port and from time interval to time interval is well within the variability inherent in the technic. Thus, for the 48-hour observation period, the gas composition remained virtually unchanged, indicating that a gaseous balance for this system can be attained.

TABLE I

THE CO_2-N_2-O_2 COMPOSITION OF ROOM AIR

DETERMINED AT VARIOUS TIMES

Sample	Percent of CO_2	Percent of N_2	Percent of O_2
1	0.095	78.035	21.870
2	0.094	78.036	21.870
3	0.082	78.261	21.657
4	0.098	78.715	21.187
5	0.087	79.819	20.094
6	0.109	78.737	21.154
7	0.099	78.388	21.513
8	0.128	78.793	21.080
$\bar{x}$	0.099	78.598	21.303
s	0.014	0.580	0.562

TABLE II

GAS COMPOSITION OF A CLOSED SYSTEM

CONTAINING ALGAE, FUNGI, AND SEWAGE ORGANISMS

TIME (hours)	PORT*	Percent of CO_2	Percent of O_2	Percent of N_2
0	1			
	2	0.09	21.09	78.82
	3	0.10	21.21	78.69
1.0	1	0.11	20.53	79.36
	2	0.11	21.02	78.87
	3	0.08	22.28	77.64
2.0	1	0.09	21.02	78.89
	2	0.11	22.20	77.69
	3	0.13	20.88	78.99
4.5	1	0.11	20.84	79.05
	2	0.11	20.82	79.07
	3	0.12	21.80	78.08
6.5	1	0.10	20.88	79.02
	2	0.11	21.06	78.83
	3	0.09	21.12	78.79
26.0	1	0.09	20.47	79.44
	2	0.09	20.47	79.44
	3	0.10	20.18	79.72
30.5	1	0.07	21.05	78.88
	2	0.11	20.90	78.99
	3	0.10	21.47	78.43
47.5	1	0.11	21.71	78.18
	2	0.12	20.35	79.53
	3	0.10	19.87	80.36
	$\bar{x}$	0.10	21.01	78.90
	s	0.013	0.610	0.625

*Port #1 from Sewage
Port #2 from Fungi
Port #3 from Algae

First Animal Run

The system was charged as described previously and a 65-gram male rat was placed in the animal chamber along with water and rat feed. Sewage degradation was followed by measuring sedimented solids with a Wintrobe tube after a 1-hour sedimentation period. Table III shows the performance of the system over a 96-hour period. The algal population exhibited a generation time of 6.5 hours (calculated from the equation, $G = \frac{0.3010t}{\log b - \log a}$). Under BRU conditions, the algal count began to peak off at 40 hours, in agreement with growth curves obtained in previous heterotrophic studies. By 72 hours, the sedimentable sewage solids had been reduced by 75 percent, indicating at least a partial degree of sewage stabilization was achieved. No measurements were made on the fungal culture for this run. Visual observation indicated growth was comparable to previously obtained growth rates.

TABLE III

PARAMETERS OF THE BRU CONTAINING ALL SUBSYSTEMS

TIME (hours)	ALGAL COUNT x 10^6/ml	SEWAGE SEDIMENT (Percent)	Percent of O_2	Percent of CO_2
0	2.64	8	20.20	0.04
4	----	-	19.10	0.08
22	25.8	7	19.10	1.20
24	----	-	12.47	7.83
28	----	-	8.43	10.04
43	92.5	7	5.77	12.32
	* Rat dead at approximately 35 hours			
72	106.8	2		
96	Sewage digested; break in system; run terminated.			

- Not observed
* Rat removed and run continued

The death of the animal at approximately 35 hours was not surprising in view of the gaseous composition within the system. Figure 4 shows the striking changes in CO_2-O_2 composition. Of particular interest is the drastic change observed between the 22nd and 24th hours. Evidently the fungal culture or the sewage system suddenly placed a large demand upon the system (or perhaps both simultaneously). The algae did not recover from this overload as evidenced by the continuation of the trend. It is doubtful if the higher CO_2 levels had a deleterious effect on the algae since, as shown in figure 5, cells grown in approximately 7 percent CO_2 grew with almost the same generation time.

Second Animal Run

Several changes were made in the BRU. Three modified Soxlet condensors were substituted in place of the flask condensors as a means of humidity control. Since the algae proved to be insufficient in gas turnover to balance the system, the small algal growth chamber was replaced with an 11.5-liter solution bottle measuring 23.8 cm by 40.7 cm. The diameter of this container

was such that, when in place, the circular fluorescent lamps touched the walls of the bottle. A fan was used to maintain temperature since air circulation was a problem. The chamber was charged with 9 liters of medium and autoclaved as described previously. The inoculum used was a 64-hour MS glucose culture of algae washed 1 time in MS. Fifty ml of this culture (concentration = 505 x 10^6 per ml) gave an initial concentration in the chamber of 2.8 x 10^6 cells per ml. Air enriched with 7 percent CO_2 was vigorously bubbled through the culture. Table IV shows the progress of the algal culture and the history of the second and third animal runs. Figure 6 represents the algal growth curve obtained during 819 hours (34 days). As shown here and in figure 4, the algal generation time was approximately 7 hours. Thus, growth within the closed BRU and that obtained with 7 percent compressed CO_2 is comparable during early growth periods.

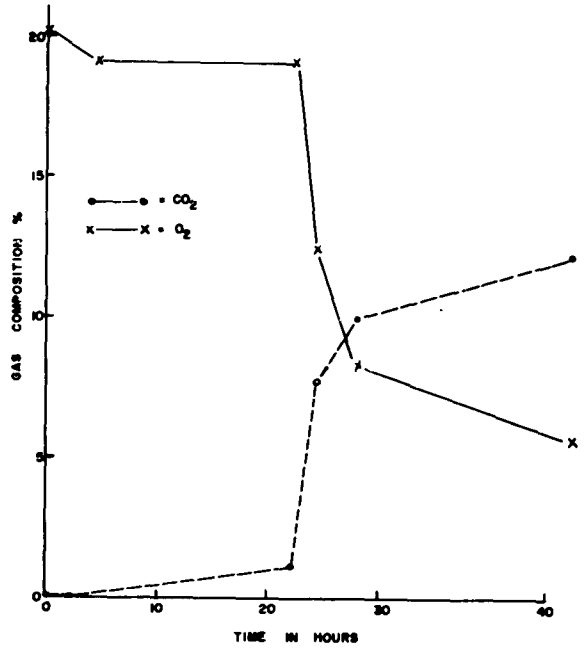

Figure 4. CO_2 and O_2 Concentrations in the BRU with All Components, First Run

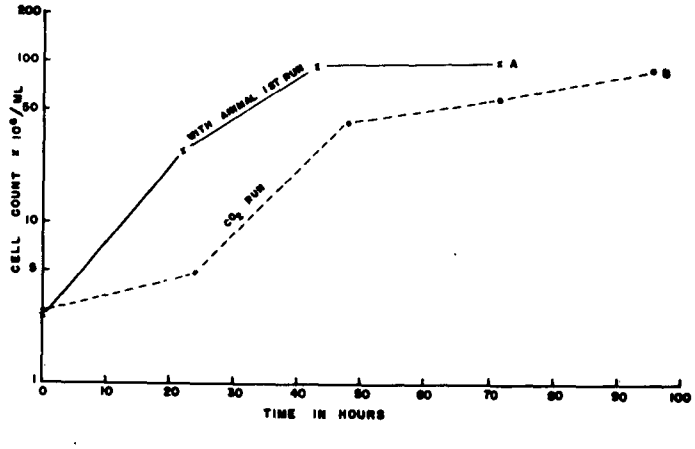

Figure 5. Algal Counts in BRU with and without Added CO_2

TABLE IV

PERFORMANCE OF THE BRU OVER AN EXTENDED PERIOD

TIME (hours)	ALGAL COUNT x 10^6/ml	Percent of CO_2	Percent of O_2	COMMENTS
0	2.8			
24	4.85			
48	41.75			
72	59.00			
96	91.50			Chemostat initiated
102	90.50			
127	85.0			
150	79.0			
174	55.8	0.104	20.83	Animal, etc., added, system closed
198	46.3	7.19	11.82	
205	62.0			Chemostat stopped
222	75.75	10.51	7.12	
229	86.75			
246	92.0	12.82	. 5.27	
254.8	104.0			1.51 liters removed from fungal chamber
270	109.13	12.25	6.28	
277	121.00			
297	126.00	9.98	10.71	
321	142.00	3.99	16.70	
342	131.75	2.61	17.95	Fresh medium added to algae
347	115.50			
365	157.25			
390	152.25			Ballast water pulled over, run terminated
	ANIMAL REMOVED, SYSTEM RUN WITH ADDED CO_2			
414	94.5			
438	117.00			
462	165.00			Feces added, medium added to fungus
	STERILITY CHECKS SHOWED ALGAE CONTAMINATED, *Pseudomonas aeruginosa* present.			
510	ANIMAL PLACED IN CHAMBER AND SYSTEM SEALED			
531		1.60	19.98	
579		1.82	18.91	
651	397.5			
675	315.0			
819		3.44	18.17	
	HEAVY VACUUM IN SYSTEM DEVELOPED, RUN TERMINATED.			

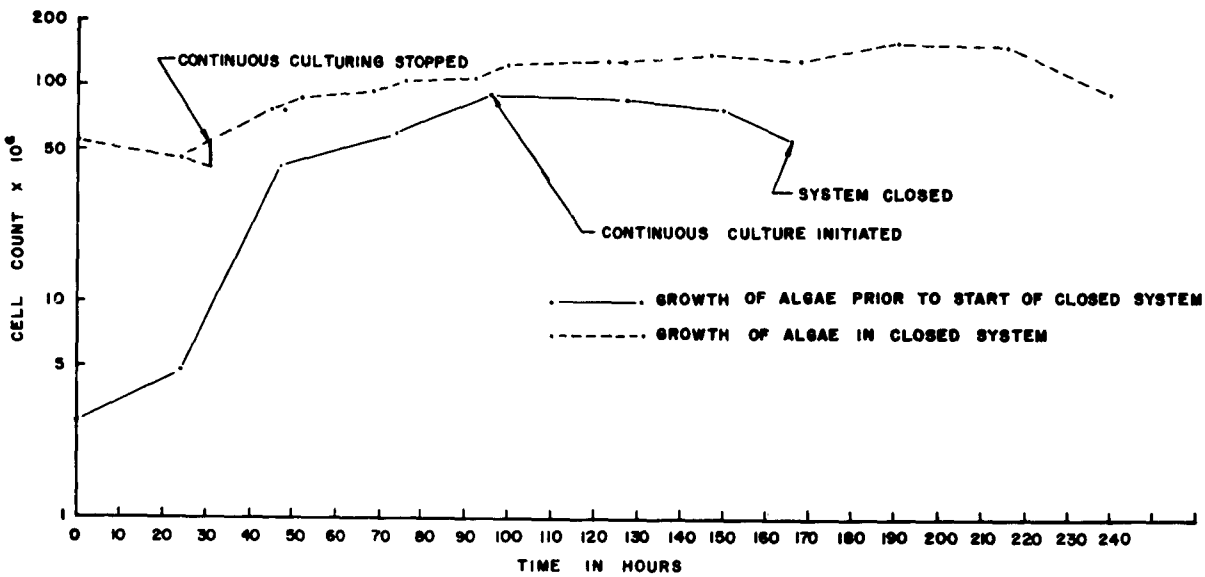

Figure 6. Algal Counts from BRU Run of 819 Hours

At 96 hours, the algal cell density reached 91.5 x 10^6 per ml and chemostat conditions were initiated. Assuming G = 8 hours, to just maintain cell density, a dilution rate of $\frac{9}{2}$ liters or 4.5 liters per 8 hours should be employed. Since it was not possible to control growth via limiting substrate concentration (chemostat technic, ref. 20), the criterion for control was cell density (turbidostat technic). The literature indicates that, although one can calculate flow rates for chemostats (turbidostats, if automatic, are self-regulating), these do not usually prove to be operationally correct. Hence, a variety of in-out flow rates were attempted. This was accomplished by pressurizing the medium reservoir and allowing drops of medium to flow into the growth chamber by gravity. Effluent flow rates as well as influent were adjusted to be equal with pinch clamps.

As shown in table IV and figure 6, attempts to maintain a constant cell density were not successful. Even when flow rates were quite low (2.5 liters per 23 hours) growth rate of the culture did not keep pace with dilution rate. Although counts were low at 174 hours, (a) a 149-gram male rat was placed in the animal chamber, (b) feces were added to the sewage chamber, (c) the fungal chamber was inoculated with an MS spore suspension, and (d) the system was closed. The initial gas analyses (by Haldane) showed a somewhat elevated CO_2 as was seen in previous runs (approximately 0.1 percent). Probably this was due to release of trapped gases in the fecal samples. Figure 7 depicts the CO_2 and O_2 levels observed in the BRU over 168 hours (7 days).

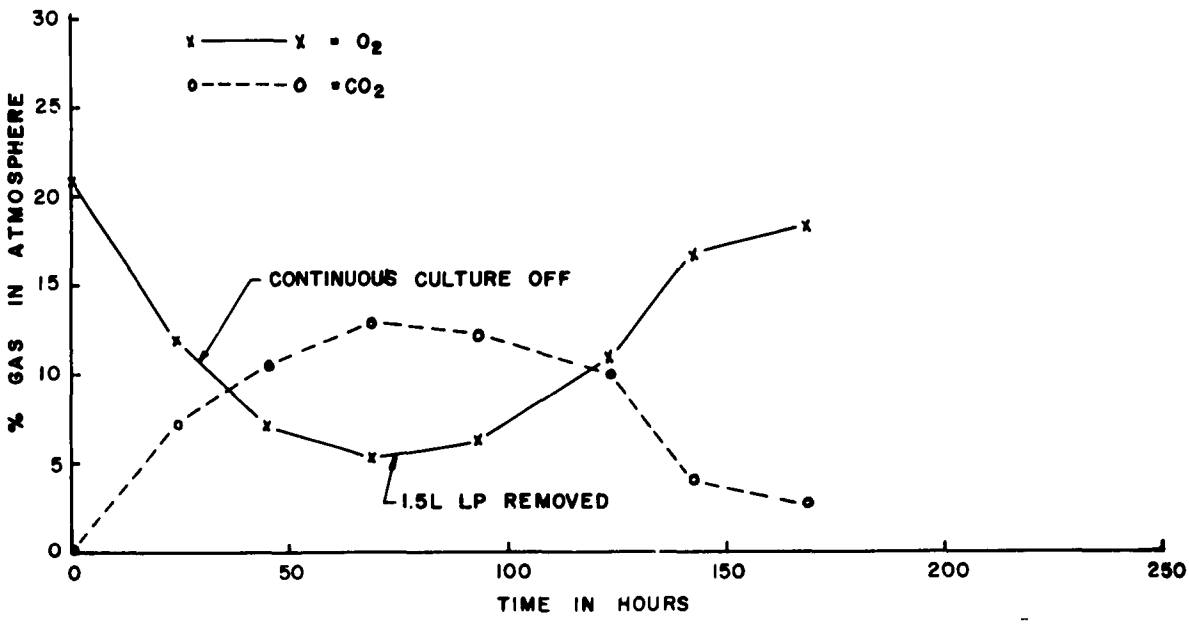

Figure 7. O_2-CO_2 Composition of Air in BRU Run of 168 Hours

After 24 hours the CO_2 had risen sharply while the O_2 had dropped. The gas curves can be described by opposite parabolic functions, the high and low points occurring at about 70 hours from the start of the experiment (246 hours in table IV). The continuous culture operation was stopped at 31 hours (205 hours in table IV), since the algal count as well as the gas balance continued to fall off. During this time of rather severe stress, the rat exhibited deep and rapid inspirations, a trace of cyanosis being observed. However, the animal continued to take food and water during this period.

At 80.5 hours, shortly after the highest CO_2 level was observed, 1.15 liters or half the volume was removed from the fungal chamber. The purpose of this was to reduce the CO_2-O_2 burden on the system, since the fungi were metabolizing excess carbohydrate and thus exhibited a high RQ. The mycelial mat was separated from the medium by filtration through cotton gauze pads, washed 3 times with distilled water, and lyophilized. A yield of 3 grams (2 grams per liter) was obtained. The nitrogen analysis of this material showed 2.4 grams of nitrogen per 100 grams or 15.2 percent protein. The gas analysis performed after the partial removal of the fungal culture showed a reverse trend: that is, the CO_2 level began to drop while the O_2 concentration began to rise. Recovery of the system (in terms of gas composition) continued until the run was terminated due to equipment breakdown. During this recovery period, the algal population continued to increase in number, the addition of fresh medium at 168 hours (342 hours in table IV) resulting in an initial drop in total count followed by an increase to a maximum level. After 216 hours (9 days) the system was shut down and the algal culture continued by bubbling CO_2-enriched air through the chamber.

Examination of the rat revealed no gross or obvious deleterious changes. Outwardly the animal had completely survived the stress and had gained weight (controls were not established). This and the previous run established the existence of an insignificant leak rate in the system, as indicated by the high CO_2-low O_2 levels observed. Thus, the basic question of gas balance has been favorably answered, in that the biological entities after a severe adjustment period did maintain this balance for a short period.

The algal culture was continued until a cell density of 165 x 10^6 per ml was reached. At this time (462 hours) feces were added to the sewage chamber and fresh medium added to the fungal chamber. Sterility checks showed the algae to be contaminated at this time. The bacterial contaminants isolated were of three types. Their descriptions and ratios are:

Organism	Count x 10^6	Percent of Total	Description
1	57	11.8	Gram (-) bacillus, colony large white
2	419	86.6	Gram (-) bacillus, colony small white
3	8	1.4	Gram (-) bacillus, colony yellow

A total of 484 x 10^6 bacteria per ml was observed. This rather large number certainly had some influence on the activity of the algal culture. Attempts were not made at this time to identify the contaminants. However, the appearance of the characteristic blue-green diffuse pigment on one of the count plates strongly suggested one of the organisms to be Pseudomonas aeruginosa.

To determine the effect of contaminated algae on the gas balance of the BRU, at 510 hours (table IV) a rat was placed in the animal chamber and the system was closed. Twenty-one hours later (531 hours, table IV) gas composition was somewhat changed but not to a great extent. Algal counts reached a maximum level of 397.5 x 10^6 per ml at 141 hours (651 hours, table IV). Thus, the contaminants seem to have a beneficial effect on the algae in that cell concentration increased by more than a factor of 2. The gas composition did not adhere strictly to desired levels; nor, in fact, did it change drastically. Therefore, although more algal cells were present, their individual activity was probably less than cells in more dilute suspensions—that is, the sum of gas exchange activity in dense cultures was not greatly different from that in less dense cultures. Certainly light could have been the limiting factor. The run was terminated 309 hours (approximately 13 days) after its inception. Thus, definite evidence is given for the general application of the system. The capability of contaminated cultures to maintain a gas balance is also indicated. After 819 hours (34 days) and a variety of environmental conditions, the algal culture was still vigorous and capable of performing its role as a gas exchange mechanism. This, along with other investigations of algae, indicates the general stability of these organisms and their capability for adapting to and functioning in a changing environment.

Ammonia Utilization by Linderina pennispora

Since the purpose of the BRU was to determine the efficacy of using a fungal culture to maintain gas and elemental balance in a closed system, the extent of NH_3 utilization by L. pennispora was determined. A major component of the fungal medium is 2 percent corn steep liquor. This material consists of a mixture of biochemical entities and is used as a source of undetermined organic requirements since its composition is unknown and probably varies from batch to batch. To determine the extent of total metabolizable substances in corn steep liquor, a simple experiment was performed. Fungal spores were inoculated into 250 ml of the medium described previously. Additional sources of nitrogen were not included. The flask was fitted with a glass wool filter to sterilize incoming air and was connected to a compressed air line. After 3 days of incubation at room temperature, the fungal mycelium was harvested. Good growth had occurred. At that time a representative, 250-ml sample of a 3-day culture was withdrawn from the BRU. This mycelium was also harvested. Both samples were washed with distilled water to remove medium constituents and then lyophilized. The sample grown in the BRU (receiving NH_3 from the sewage degradation) weighed 0.5287 gram, while the sample grown without added nitrogen (except for that in the corn steep liquor) weighed 0.3996 gram. Nitrogen analysis (by Kjeldahl) was performed on these samples and on corn steep liquor. The corn steep liquor gave a value of 3.97 grams of nitrogen per 100 ml. Therefore, the amount of nitrogen added to the 250 ml of medium can be calculated:

$$\frac{3.97 \text{ gm}}{100 \text{ ml}} = \frac{x \text{ gm}}{0.41 \text{ ml}}$$

(The figure of 0.41 ml was the volume of corn steep liquor added to the medium.)

$x = 0.01628$ gm N/250 ml medium

Nitrogen content of the fungal samples was:

1 - BRU grown (+ NH_3) = 3.078 mg/100 mg

2 - Non-BRU grown (-NH_3) = 4.514 mg/100 mg

Converting to protein (assuming all nitrogen is protein):

1 = 19.2 percent 2 = 28.2 percent

The total nitrogen recovered from each sample was calculated:

1 - (BRU grown): $\frac{3.078}{100} = \frac{x}{0.5287}$; $x = 0.01627$ mg/250 ml

2 - (Non-BRU grown): $\frac{4.514}{100} = \frac{x}{0.3996}$; $x = 0.01804$ mg/250 ml

Comparing these values with the nitrogen content of corn steep liquor, nitrogen utilization in the form of NH_3 did not seem to occur, or at least it was not stimulatory. That is, assuming the value of 0.01804 is not statistically different from 0.01628, the corn steep liquor provided the total source of nitrogen in the BRU. Also of interest is the fact that the BRU sample gave a lower nitrogen content but grew to a greater extent (by about 20 percent). This is consistent with Gray's findings* that, as L. pennispora grows after a 2-day period, the total protoplasm increases while nitrogen remains constant for the culture and hence diminished per unit of protoplasm.

DISCUSSION

The use of a complex biological system to maintain a quasi-energy and elemental balance in a closed ecology must be justified on two accounts: reliability equal or superior to physicochemical methods and regenerative capability commensurate with the degree of closure of the total system. We normally consider biological subsystems as necessary when logistics require an endogenous supply of substrate, i.e., breathable atmosphere and nutritional support.

The system reported herein was devised primarily to supplement or perhaps even circumvent using algae as a food source for man since these organisms possess many undesirable characteristics. [Several workers (refs. 11, 19, 22, 25, 28, 36, 43, 44) have provided evidence for some nutritional value of algae. However, problems in nutritional quality as well as acceptability have been encountered.] W. D. Gray* has studied the application of various fungal entities as converters of carbohydrate to protein for human consumption. Some of these organisms, particularly L. pennispora, have the ability to obtain nitrogen from NH_3. Since this gas is a product of aerobic sewage degradation, and in view of the nutritive potential of the Deuteromycetes, the investigation of a biological system comprised of four entities was warranted.

Although no one aspect of the functionality of any particular biological subsystem can be deemed as of prime importance, the maintenance of the requisite gas composition is a good starting point. The experiments described in this report, although certainly not definitive, lend support to the possibility of controlling atmospheric make-up through the combined activities of several biotic forms. The results indicate that, although optimum conditions (20 percent O_2, 0.5 percent CO_2)

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*Gray, W. D., Personal Communication, Ohio State University, Columbus, Ohio.

were not met, by adjusting metabolic rates of the microorganisms concerned a gaseous balance could be obtained. Under the experimental conditions, the fungal culture did not contribute to the system balance. Probably it added to the CO_2 burden observed. However, the limited nutritional study indicates that a nonnitrogen (or limited) containing medium is necessary to capitalize on the "NH_3-fixing" properties of the fungus. This necessitates the initiation of studies on the minimal nutritional requirements of L. pennispora and the formulation of a medium which will allow growth at a level controlled by the concentration of effluent NH_3. In addition, the O_2-CO_2 burden on the system should, of course, be minimal.

The above discussion is a rather oversimplification of the problem. Assuming the fungus can provide adequate nutritional support for man, in conjunction with the algae, a material balance as depicted in figure 1 must be maintained. Evidence to date is not favorable in this respect. Problems of accumulation of unused substrate—such as incompletely degraded sewage, undigested algal residues, and undigested food residues—are evident.

The reutilization of human wastes is sometimes considered unnecessary and perhaps impossible to the extent required. Calculations by Johnson* indicate that 25 kg of dry milk solids can supply a man for a year with the materials which could be made available from feces (over a 1-year period). Should this figure be correct, serious consideration must be given to using stored material in conjunction with a physicochemical treatment of the feces. Removal of water and reduction to a storable material might then be a more suitable approach. Rigorous calculations, based on accurate data, must be made of the trade-off point at which such an approach is impractical. Such considerations must include the physical parameters of the systems—i.e., biological waste treatment and recycling versus storage of treated wastes (to include equipments) and nutrients. At first glance, a completely bioregenerative system seems most suitable for a permanent lunar base consisting of about 30 men. Golueke, Oswald, and McGauhey (ref. 14) have presented some estimates of closed system logistics, based on experimental data. They suggest that from 304 to 411 pounds of equipment, to include gas exchange and waste treatment, can support a man for 3 weeks. Since biological systems become more effective with extensions in mission time and crew size, large permanent space stations will probably require biological control systems. However, the extent of closure required for this type of ecosphere must be governed by such nonbiological considerations as: (a) length of tour of duty, (b) incidence of physical communication, and (c) cost of a periodic earth-to-lunar base supply system. If it becomes feasible and necessary for frequent contacts with the extraterrestrial site, the degree of closure of the "closed system" can be lessened. Thus, the requirement for theoretically 100 percent bioregenerative efficiency can be considerably decreased.

On the other hand, if bioengineering data indicates that a considerable degree of closure of the "closed ecology" is mandatory, certain physicochemical methods for waste degradation and subsequent turnover should be considered. The Zimmerman wet oxidation method (ref. 8) yields end products which appear suitable for reutilization by biological entities within the system. These end products consist of CO_2, inorganic salts,* and possibly organic acids, the particular composition depending upon starting material and the degree of oxidation obtained. The applicability of this technic has not been proved. Intensive research is required to evaluate its potential for closed system use.

The Atomized Suspension Technic (AST) is a high-temperature process which has the distinct advantage of a batch operation (as opposed to continual) capability (ref. 12). The products of the process are rather highly pyrolized and thus are conceivably somewhat more easily reutilized by the bioforms within the closed system. Both of these approaches must of course be considered from biological, engineering, and logistic standpoints. At the present time no single approach to

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*Johnson, R. E., Personal Communication, University of Illinois, Urbana, Illinois.

* Johnson, M. J., Personal Communication, University of Wisconsin, Madison, Wisconsin.

waste processing (and possible recycling) has been proved practical. The problem then resolves itself into three alternatives: (a) biological decomposition with recycling, (b) physicochemical decomposition with recycling, or (c) storage with or without some treatment (such as dehydration). Selection of the approach is dependent upon the most favorable compromise of reliability, weight and volume, matching of AQ's and RQ's, and total system demands (rates, volumes, concentrations, etc.).

The significant role of a fungal organism in a closed or semiclosed ecology is that of nutritional support. The potential of these organisms for space systems has been aptly described by Gray (ref. 16). He discusses the quality of fungal protein and suggests that manipulation of environmental parameters could yield protein of the requisite chemical composition. The work with algae has been less promising in this respect. Therefore, the added complexity of a fungal subsystem in the closed ecosphere can only be justified if two conditions are adequately fulfilled: (a) the waste materials arising in the closed system are in a form or can be efficiently converted to a material readily accessible to fungal metabolism and (b) the nutritive value of the specific fungal entity employed growing under the specific conditions established by a closed system is adequate to meet at least the minimal protein requirements of man. Possibly the inclusion of an "intermediary metabolizer" or converter is warranted if the lessened efficiency of a longer food chain can be tolerated.

CONCLUSIONS

1. Based on the limited data obtained in these experiments, several microbial entities can be incorporated into a closed system with maintenance of the requisite gas balance. With the fortunate choice of a rat which tolerated extreme CO_2-O_2 conditions, the capability of the organisms involved to acclimate to and readjust the gas composition was observed.

2. The gas exchange rates of the algal component within the experimental BRU were far from adequate. Light intensity per total culture was certainly the limiting factor. The problem of optimal design for algal gas exchangers is far from resolution. In addition, since continuous growth conditions are required, information on macronutrient utilization rates is required so that an elemental balance within the total system can be attained.

3. L. pennispora exhibits a preference for nitrogen in the organic form. This is in agreement with the findings of Gray.* Hence, using corn steep liquor as an accessory factor(s) source is negated and an investigation is required of those parameters necessary for maximum NH_3 utilization.

4. Since the major justification for including fungi within a closed system concerns their nutritional utility, a rigorous determination of the nutritive value of these organisms must be accomplished. This should include not only animal digestibility trials, protein efficiency ratio, caloric value, etc., but also biochemical data on the composition of the fungal protoplasm. In particular, attention should be directed to amino acid analysis, as well as specific carbohydrate and lipid content.

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*Gray, W. D., Personal Communication, Ohio State University, Columbus, Ohio.

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Unclassified report

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animal chamber containing a white adult male rat. Although many difficulties were encountered, this bioregenerating unit could be operated under closed conditions for 390 hours. Gas analyses indicated CO_2 and O_2 levels can be maintained at desirable levels. Material balance within the system was not attempted. However, nitrogen analysis of the resultant fungal mass showed that data on the basic physiology of the organism is required before such a balance can seriously be attempted. The relationship between mission requirements and the level of biological complexity of a closed ecological system was discussed.

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